Assessment of β_2 - and β_3 -Adrenoceptors in Rat White Adipose Tissues by Radioligand Binding Assay

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We investigated the characteristics of β -adrenoceptors (β -ARs) in rat white adipose tissues (WAT) with a radioligand receptor binding assay using (–)-[³H]-CGP12177. Scatchard analysis revealed that there are high- and low-affinity sites for (–)-[³H]-CGP12177 in WAT. The (–)-[³H]-CGP12177 bound to a high-affinity site was displaced by 1 μ M propranolol. The rank of pK_i values of catecholamines for the site was isoproterenol > epinephrine > norepinephrine. By contrast, BRL37344A, BRL35135A and SR59230A, β_3 -selective agonists had high affinity for the low-affinity site of (–)-[³H]-CGP12177, whereas (–)-[³H]-CGP12177 bound to a low-affinity site was completely displaced by 100 μ M bupranolol but not 1 μ M propranolol. The pK_i values of the catecholamines (isoproterenol, norepinephrine, epinephrine) for this site were very low. In addition, the correlation between the pK_i values of various β -agonists for the low-affinity site of rat WAT and those obtained from rat cloned β_3 -ARs was significant, but those of human cloned β_3 -ARs were not. Consequently, the results suggested that the high- and low-affinity sites were β_2 -ARs and β_3 -ARs in rat WAT, respectively.

Key words β_3 -adrenoceptor; [³H]-CGP12177 binding; rat white adipose tissue

There is increasing evidence that β -adrenoceptors (ARs) can be classified into at least three subtypes. Those are typical β_1 - and β_2 -ARs and β_3 -ARs. The β_3 -ARs were first discovered in rat white adipocytes by Harms *et al.*¹⁾ as atypical β -ARs, then Tan and Curtis-Prior²⁾ proposed that they should be described as β_3 -ARs. Subsequently, the genes encoding human β_3 -ARs were isolated by Emorine *et al.*³⁾ β_3 -ARs were found in various tissues, for example in adipose tissue,^{4–7)} intestinal tract smooth muscle,^{8–11)} heart,^{12,13)} vascular smooth muscle,^{14,15)} skeletal muscle,^{16,17)} liver,¹⁸⁾ and airway smooth muscle.^{19,20)} β_3 -ARs participate in the modulation of glycerol release, thermogenesis and glycometabolism; therefore, selective agonists for β_3 -ARs may be useful in treating metabolic disturbances such as obesity and diabetes in which weight control is difficult. Details of the pharmacological properties of β_3 -ARs, however, are unknown. To evaluate the coexistence of β -ARs and the binding characteristics of [³H]-CGP12177 to β_3 -ARs in adipose tissues is important and would be useful in assessing the affinity of new remedies.

The ligands [³H]dihydroalprenolol and [¹²⁵I]iodocyanopindolol have been used to study adipocyte β -ARs. They are highly lipophilic and membrane-permeable, so it is possible that they labeled not only surface, but also internal receptors. Therefore, assessments using these lipophilic radioligands might not necessarily reflect the characteristics of the receptors on the cell surface. On the other hand, [³H]-CGP12177, which is a partial agonist of β_3 -AR, is highly hydrophilic and membrane-impermeable.²¹⁾ In addition, Lönnqvist *et al.*²²⁾ have suggested that CGP12177 is a better tool for studying β_3 -ARs than BRL37344A, which is a selective β_3 -agonist.²³⁾ Therefore, it is considered that [³H]-CGP12177 is the most reliable radioligand for assessing β -ARs on the adipocyte surface.^{24–26)}

Thus, we clarified the binding characteristics of (–)-

[³H]-CGP12177 to β_3 -ARs of membranes in rat white adipose tissues (WAT) by Scatchard analysis and competition binding studies.

MATERIALS AND METHODS

Drugs and Chemicals (–)-[³H]-CGP12177 (Amersham), (+)-isoproterenol and (–)-epinephrine (Sigma Chemical Co., U.S.A.), (–)-norepinephrine, serotonin, histamine and atropine (Wako Pure Chemical Industries, Ltd., Japan), yohimbine (Nacalai Tesque Inc., Japan) and butoxamine (Burroughs Wellcome Co., U.S.A.) were purchased. The following drugs were donated: BRL37344A ((±)-4-(2'-(2-hydroxy-2-(3-chlorophenyl)ethylamino)propyl)phenoxyacetic acid sodium salt sesquihydrate) and BRL35135A ((±)-methyl-4-(2-(2-hydroxy-2-(3-chlorophenyl)ethylamino)propyl)phenoxyacetate hydrobromide) (SmithKline Beecham Pharmaceuticals, England), SR59230A (3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2*S*)-2-propanol oxalate) (Sanofi Midy S.P.A. Research Center, Italy), bupranolol hydrochloride (Kaken Pharmaceuticals Co., Ltd., Japan), betaxolol (Mitsubishi Kasei Co., Ltd., Japan), metoprolol (Ciba-Geigy Co., Ltd., Japan), nadolol (Dainippon Pharmaceuticals, Ltd., Japan), atenolol hydrochloride, ICI-118551 and propranolol (ICI Pharmaceuticals, Japan) bopindolol, pindolol, 18-502 (4-(3-*tert*-butylamino-2-hydroxypropoxy)-2-methylindole) and 20-785 (4-(3-*tert*-butylamino-2-hydroxypropoxy)indol-2-carboxylic acid) (Sandoz Pharmaceuticals, Ltd., Switzerland).

Preparation of Membrane-Enriched Fraction from the Rat WAT Membrane-enriched fractions were prepared as described.¹⁶⁾ Male Wistar rats weighing about 350 to 450 g were killed by exsanguination and WAT were immediately removed. WAT was then minced with scissors and homogenized in 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EGTA at 23 °C with a Polytron

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homogenizer.

The homogenates were centrifuged for 10 min at $1000 \times g$ at 23°C . The supernatant was filtered through triple layers of surgical gauze. The filtrates were again centrifuged for 15 min at $10000 \times g$. The supernatant was transferred to ultracentrifuge tubes and centrifuged for 30 min at $100000 \times g$. The supernatant was discarded, then the pellets were resuspended in ice-cold buffer (50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , 1 mM EGTA). After a second centrifugation for 30 min at $100000 \times g$, the pellet was resuspended in the incubation buffer (50 mM Tris-HCl (pH 7.4), 10 mM MgCl_2 , 0.15 M NaCl) and stored at -80°C .

The concentration of membrane protein was determined using bovine serum albumin as a standard according to Lowry *et al.*²⁷⁾

Radioligand Binding Assay A) Scatchard Analysis: Saturation experiments were performed in duplicate with (–)-[^3H]-CGP12177 in the presence (non-specific binding) or absence (total binding) of 1 mM propranolol. In brief, a membrane suspension (20 μg of protein) was incubated for 3 h at 23°C with various concentrations (0.1–40 nM) of (–)-[^3H]-CGP12177 in a total volume of 500 μl . At the end of the incubation period, the incubation medium was immediately filtered through a Whatman GF/C glass fiber filter as described by Satoh *et al.*²⁸⁾ The radioactivity level was counted with a liquid scintillation counter (Packard 2200CA). The difference in mean values between the total and non-specific binding was taken as the specific binding.

B) Displacement Experiments: Displacement experiments were performed in duplicate with 10 nM of (–)-[^3H]-CGP12177 in the presence of various concentrations of β - and β_3 -agonists. At the end of the incubation period, the incubation medium was immediately filtered through a Whatman GF/C glass fiber filter. The radioactivity level was counted with a liquid scintillation counter. The difference in mean values between the total and non-specific binding was taken as the specific binding.

In the saturation experiments, 1 μM propranolol, 100 μM bupranolol, and 100 μM prazosin and yohimbine (α -blocker) or serotonin was added to the reaction mixture 20 min before the addition of (–)-[^3H]-CGP12177.

Data Analysis All kinetic analyses were performed as described by Satoh *et al.*²⁸⁾ Statistics were analyzed by Student's *t*-test.

RESULTS

Scatchard Analysis The Scatchard plot was curvilinear, indicating that there are at least two different affinity sites for (–)-[^3H]-CGP12177 (Fig. 1A). The K_d values of the high- and low-affinity sites were 0.42 ± 0.06 and 40.42 ± 10.77 nM, and the B_{max} values were 321.0 ± 21.7 and 3655.1 ± 454.3 fmol/mg protein, respectively. Although 1 μM propranolol completely displaced (–)-[^3H]-CGP12177 binding for the high-affinity site, it did not affect the binding for the low-affinity site (Fig. 1B). The K_d and B_{max} values of the low-affinity site were not significantly different from those obtained from the

Fig. 1. Scatchard Plots of (–)-[^3H]-CGP12177 Binding to Rat WAT

Scatchard Plots of (–)-[^3H]-CGP12177 binding to rat WAT without displacements (A) or in the presence of 1 mM propranolol (B) and 100 μM bupranolol (C) as displacers.

Table 1. The pK_i Values of β -Agonists and Antagonists in Rat White Adipose Tissues

Drugs		pK_i values	
		High-affinity site	Low-affinity site
Agonists			
Isoproterenol	(5)	10.86 ± 0.55	$4.42 \pm 0.20^*$
Epinephrine	(7)	9.22 ± 0.32	$3.62 \pm 0.22^*$
Norepinephrine	(6)	8.34 ± 0.59	$3.88 \pm 0.39^*$
BRL37344A	(5)	4.82 ± 0.20	$8.89 \pm 0.52^*$
BRL35135A	(5)	4.79 ± 0.22	$10.07 \pm 0.87^*$
SR59230A	(5)	5.14 ± 0.22	$9.47 \pm 0.70^*$
Antagonists			
Propranolol	(5)	8.61 ± 0.48	$4.41 \pm 0.50^*$
Pindolol	(4)	8.53 ± 0.15	$2.71 \pm 0.23^*$
ICI118551	(5)	9.37 ± 0.34	$3.44 \pm 0.26^*$
Arotinolol	(4)	8.83 ± 0.25	$4.17 \pm 0.39^*$
Betaxolol	(4)	6.80 ± 0.43	$3.06 \pm 0.42^*$
Bopindolol	(4)	9.51 ± 0.83	$4.87 \pm 0.61^*$
Alprenolol	(6)	8.84 ± 0.38	$4.10 \pm 0.40^*$
Nadolol	(7)	8.67 ± 0.50	$3.79 \pm 0.21^*$
Metoprolol	(6)	8.28 ± 0.60	$3.87 \pm 0.13^*$
Butoxamine	(5)	8.31 ± 0.47	$3.40 \pm 0.26^*$
Bupranolol	(6)	9.53 ± 1.95	$6.17 \pm 0.42^*$
CGP12177	(4)	9.11 ± 0.30	$2.92 \pm 0.08^*$
18-502	(4)	10.60 ± 0.17	$5.04 \pm 0.73^*$
20-785	(4)	8.07 ± 0.84	$4.22 \pm 0.37^*$

These data represent mean $\pm$ S.E. of 4–7 experiments. The concentrations of (–)-[3 H]-CGP12177 were 10 nM for most drugs and 5 nM for betaxolol, metoprolol and butoxamine. * $p < 0.01$; high- vs. Low-affinity site.

Scatchard plots shown in Fig. 1A. On the other hand, 100 μ M bupranolol completely displaced (–)-[3 H]-CGP12177 binding for the high- and the low-affinity sites (Fig. 1C).

Displacement Experiments The competition activities of various agonists against 10 nM (–)-[3 H]-CGP12177 were examined. The pK_i values of isoproterenol, norepinephrine, epinephrine, BRL37344A, BRL35135A and SR59230A are shown in Table 1. For the high-affinity site, the rank order of pK_i values of catecholamines was isoproterenol (10.86 ± 0.55) > epinephrine (9.22 ± 0.32) > norepinephrine (8.34 ± 0.59), which corresponded to that of β_2 -ARs. On the other hand, higher pK_i values of each antagonist for high-affinity sites than those for the low-affinity site were observed (Table 1). In addition, the pK_i value of bupranolol for the low-affinity site was 6.17 ± 0.42 , and this value of this drug to the low-affinity site was the highest among the β -blockers tested here.

The IC_{50} values of prazosin, yohimbine and serotonin against high- and low-affinity sites were more than 1 mM (data not shown).

Relationships between the pK_i Values of β -Antagonists Obtained from Rat WAT and from β_1 - and β_2 -ARs We have reported on the pharmacological characteristics of β_1 - and β_2 -ARs in rat heart and bovine trachea.^{28–31} The pK_i values of β -antagonists in this study were compared with the values in our previous studies. The correlations between the pK_i values of various β -antagonists for the high-affinity sites for (–)-[3 H]-CGP12177 in rat

Fig. 2. Correlation of pK_i Values of β -Antagonists between that for the High-Affinity Site of (–)-[3 H]-CGP12177 in WAT and for β_2 - or β_1 -ARs in Rat Heart or Bovine Trachea

The horizontal axis represents the pK_i values of β_2 (A, B, C and D)-ARs in rat white adipose tissues and the vertical axis represents β_2 (A and C)- or β_1 (B and D)-adrenoceptors in rat heart or bovine trachea. Each point was represented as 1, propranolol; 2, pindolol; 3, ICI118551; 4, arotinolol; 5, bopindolol; 6, alprenolol; 7, l-metoprolol; 8, butoxamine and 9, betaxolol; respectively. A) $Y = 0.72X + 1.56$, $r = 0.52$, B) $Y = 0.18X + 6.23$, $r = 0.14$, C) $Y = 1.03X - 1.31$, $r = 0.77$, D) $Y = 0.23X + 5.28$, $r = 0.20$. Data were taken from Sakuma *et al.*²⁹ and Tsuchihashi *et al.*³⁰ (A and B); and Hosohata *et al.*³¹ and Satoh *et al.*²⁸ (C and D), respectively.

Fig. 3. Correlation of pK_i Values of β -Antagonists between that for the Low-Affinity Site of (–)-[3 H]-CGP12177 in WAT and for β_2 - or β_1 -ARs in Rat Heart or Bovine Trachea

The horizontal axis represents the pK_i values of β_3 (A, B, C and D)-adrenoceptors in rat white adipose tissues and the vertical axis represents β_2 (A and C)- or β_1 (B and D)-adrenoceptors in rat heart or bovine trachea. Each point was represented as 1, propranolol; 2, pindolol; 3, ICI118551; 4, alprenolol; 5, bopindolol; 6, alprenolol; 7, l-metoprolol; 8, butoxamine and 9, betaxolol, respectively. A) $Y=0.57X+5.63$, $r=0.31$, B) $Y=0.38X+6.31$, $r=0.22$, C) $Y=1.29X+2.51$, $r=0.61$, D) $Y=0.64X+4.69$, $r=0.36$. Data were taken from Sakuma *et al.*,²⁹ 1991 and Tsuchihashi *et al.*³⁰ (A and B); Hosohata *et al.*³¹ and Satoh *et al.*²⁸ (C and D), respectively.

WAT and the values for β_2 -ARs were high (correlation coefficient (r)=0.52 and 0.77 (Fig. 2A and B), but the values for β_1 -ARs (r =0.14 and 0.20 (Fig. 2C and D)) in rat heart and bovine trachea were not. Furthermore, there was no correlation between the pK_i values of various β -antagonists for the low-affinity sites for (–)-[3 H]-CGP12177 in rat WAT and the values for rat heart β_2 -ARs (r =0.31, Fig. 3A) and β_1 -ARs (r =0.22, Fig. 3B). These results suggest that high-affinity sites for (–)-[3 H]-CGP12177 are dominantly β_2 -ARs.

Relationships between the pK_i Values of β -Agonists Obtained from Rat WAT and from Cloned β_3 -ARs The pK_i values of isoproterenol, norepinephrine, epinephrine and BRL37344A in rat WAT were compared with those for β_3 -ARs cloned from rats and humans and with activities (pK_{act}) of the β -agonists on cAMP accumulation in the CHO cells expressing cloned β_3 -ARs. The compared data were taken from Blin *et al.*³² (Fig. 4A), Liggett³³ (Fig. 4B), and from Muzzin *et al.*³⁴ (Fig. 4C). The pK_i values of the agonists for the low-affinity site of (–)-[3 H]-CGP12177 in rat WAT significantly correlated with those obtained from rat cloned β_3 -ARs, with r -values of 0.96 ($p<0.05$), 1.00 ($p<0.01$) and 0.98 ($p<0.05$), respectively. On the other hand, there was no significant relationship between rat WAT and human cloned β_3 -ARs, compared with the data taken from Blin *et al.*³² (Fig. 4D) and from Liggett³³ (Fig. 4E). In addition, the pK_i values of these drugs for the low-affinity site in rat WAT

correlated better with the pK_{act} values obtained from cells expressing rat cloned β_3 -ARs with r -values of 0.97 ($p<0.05$) and 0.80 (Fig. 5A and B), than those obtained from cells expressing human β_3 -ARs (Fig. 5C), according to data taken from Nahmias *et al.*³⁵ (Fig. 5A and C) and Muzzin *et al.*³⁴ (Fig. 5B), respectively.

DISCUSSION

It has been reported that β_1 -AR predominantly locates with small amounts of β_2 -AR on rat brown and white adipose membranes,⁵ that additional atypical β -ARs subtype named β_3 -ARs³ locates in colon smooth muscle and the hearts in addition to white and brown rat fat cells, and that β_1 -, β_2 - and β_3 -ARs locate in dog fat cells.⁵

In this study, the Scatchard plots indicated that there are at least two binding sites for (–)-[3 H]-CGP12177, and the K_d values of the low-affinity site were 100 times higher than that of the high-affinity site. The results show that there are at least two β -AR subtypes in rat WAT. It is suggested that (1) the high-affinity sites for (–)-[3 H]-CGP12177 are dominantly β_2 -ARs, because they were inhibited by 1 μ M propranolol, a blocker against β_1 - and β_2 -ARs, and correlations of pK_i values of β -antagonists between in rat WAT and in other tissues were high, and that (2) the low-affinity sites for (–)-[3 H]-CGP12177 are β_3 -ARs, because they were not inhibited completely by 1 μ M propranolol but almost completely inhibited by

Fig. 4. Correlation of pK_i Values of β -Agonists between that for the Low-Affinity Site of $(-)-[^3H]$ -CGP12177 and for β_3 -ARs Cloned from Rat or Human Adipocytes

The horizontal axis represents the pK_i value of β_3 -ARs in rat white adipose tissues. The vertical axis represents the pK_i values of rat (A, B and C) or human (D and E) cloned β_3 -ARs. Each point is represented as 1, isoproterenol; 2, norepinephrine; 3, epinephrine and 4, BRL37344A, respectively. A) $Y = 0.20X + 4.75$, $r = 0.96$ ($p < 0.05$); B) $Y = 0.61X + 1.72$, $r = 1.00$ ($p < 0.01$); C) $Y = 0.58X + 1.49$, $r = 0.98$ ($p < 0.05$); D) $Y = 0.19X + 4.96$, $r = 0.55$; E) $Y = 0.32X + 3.12$, $r = 0.85$. Data were taken from reports of Blin *et al.*,³²⁾ (A and D), Liggett³³⁾ (B and E) and Muzzin *et al.*,³⁴⁾ (C), respectively.

100 μM bupranolol, a blocker against β_1 -, β_2 - and β_3 -ARs at the micro molar order.^{8,12,15)} Arch *et al.*²³⁾ reported that BRL37344A has more potent anti-obesity action in rats, hamster and dog fat cells than isoproterenol. In addition, Galitzky *et al.*⁵⁾ have shown that in dog adipose tissues, the β_3 -AR subtype was stimulated only at high catecholamine concentrations. It is because of the differences in the chemical structure of BRL37344A and BRL35135A from catecholamines that these β_3 -agonists have a highly lipophilic Cl-substituent on the catechol ring and bulky 1-(4-methoxybenzyl)ethyl derivatives on the amine substituent. The specificity for β_3 -ARs of these compounds might be attributed to their unique structures. BRL37344A, BRL35135A and SR59230A, which are also β_3 -agonists, showed high affinity for the low-affinity site of $(-)-[^3H]$ -CGP12177 in rat WAT. And isoproterenol ($pK_i = 4.42$), epinephrine ($pK_i = 3.62$) and norepinephrine ($pK_i = 3.88$) had low affinity for the low-affinity site of

$(-)-[^3H]$ -CGP12177 in rat WAT, in agreement with the studies by Muzzin *et al.*³⁴⁾ using Chinese hamster ovary (CHO) cells expressing cloned β_3 -ARs. The correlations between the pK_i values obtained in rat WAT and the pK_i values obtained from rat cloned β_3 -ARs or pK_{act} values of β -agonists and BRL37344A obtained from CHO cells expressing rat cloned β_3 -ARs were high. Thus, these present results suggest that the low-affinity sites for catecholamines are β_3 -ARs in rat WAT. Additionally, the 100 μM α -blockers (prazosin and yohimbine), serotonin, histamine and atropine, had no effect on $(-)-[^3H]$ -CGP12177 binding to high- and low-affinity sites for $(-)-[^3H]$ -CGP12177 in rat WAT as assessed by Scatchard analysis and displacement studies. These results suggested that $(-)-[^3H]$ -CGP12177 binding sites in the high- and low-affinity site of rat WAT were not α -ARs, serotonin, histamine and muscarine receptors, but β -ARs. The present data show that only β_3 -selective agonists have

Fig. 5. Correlation between the pK_i Values of β -Agonists for the Low-Affinity Site of $(-)-[^3H]$ -CGP12177 and the pK_{act} Values of β -Agonists on cAMP Accumulation via Cloned β_3 -ARs

The horizontal axis represents the pK_i values of β_3 -ARs in rat white adipose tissues and the vertical axis represents the cAMP accumulation activity (pK_{act}) of rat (A and B) or human (C) cloned β_3 -ARs. Each point represents: 1, isoproterenol; 2, norepinephrine; 3, epinephrine and 4, BRL37344A. A) $Y=0.30X+6.74$, $r=0.97$ ($p<0.05$); B) $Y=0.35X+5.95$, $r=0.80$; C) $Y=0.07X+7.66$, $r=0.37$. Data were taken from Nahmias *et al.*³⁵ (A and C) and Muzzin *et al.*³⁴ (B), respectively.

high selectivity for low affinity sites for $(-)-[^3H]$ -CGP12177 in rat WAT as well as human and rat cloned β_3 -ARs (Figs. 4 and 5). This supports that this assay system using rat WAT is useful for estimating the β_3 -selectivity of various drugs. Other merits of this assay system are: (1) this system using rat WAT more closely resembles the physiological state than that transfectants; (2) it is possible to compare the selectivity of various drugs for a β_3 subtype with that for other β subtypes in this assay.

The correlations between the pK_i values themselves and between the pK_i values and pK_{act} values were evident between the same species, but not between the pK_i values of rat WAT vs. the pK_i and pK_{act} values for human cloned β_3 -ARs (Figs. 4 and 5), although rat WAT β -ARs have selectivity for the β_3 agonist. These receptors might have common core sites among species that accept β_3 -selective ligands, and have different modulation sites that are peculiar to species and influence ligand binding profiles. It might be possible to deduce the three-dimensional structures of these receptors by integration and comparison of the information about their structure-activity relationships, the three-dimensional structure of various β -AR selective agents, and the amino acid sequence of the receptors. As Lafontan *et al.*^{25,26} suggested, each subtype pharmacologically differs species- and tissue-specifically.

It is considered that β_3 -AR selective agonists could be used to treat not only metabolic disorders such as obesity or diabetes in which weight control is difficult, but also heart failure and bronchial diseases during resistance to typical β -agonists.^{12,15,26,36} Thus, the assessment of

binding to β_2 - and β_3 -ARs by agonists is important and useful in assessing their use in treatment.

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